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Quercetin attenuates chronic ethanol hepatotoxicity: Implication of "free" iron uptake and release

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ABSTRACT

Emerging evidence has displayed that oxygen free radicals especially ones promoted by "free" iron play an important role in the development of alcoholic liver disease (ALD). Naturally-occurring quercetin has been reported to prevent ALD and iron overload-induced damage aside from the "free" iron. The purpose was to explore the potential mechanisms by which quercetin arrests alcohol-induced "free" iron disorder. Chronic alcohol (30% of total calories) or iron (0.2%)-fed adult male C57BL/J mice for 15 weeks resulted in significantly elevated levels of hepatic iron, labile iron pool-Fe and serum non-transferrin bound iron, accompanied with sustained oxidative damage. The hepatotoxicity was further exacerbated by ethanol and iron. Quercetin (100 mg/kg. body weight) alleviated the detrimental effects induced by ethanol and/or iron. The expressions of divalent metal transporter 1, zinc transporter member 14, mucolipin 1, transferrin receptor 1 (TfR1) and ferritin were up-regulated by ethanol and/or iron, which were partially normalized by quercetin. Quercetin prevented ethanol-induced hepatotoxicity, which may be partially attributed to the alleviated disorder of bound iron and "free" iron. The significant suppression of ethanol-stimulated molecules for "free" iron uptake and release may contribute to the hepatoprotective effect of quercetin, although TfR1-mediated physiological pathway of iron uptake also played a role.

1. Introduction

Excessive alcohol consumption, contributed to 4.6% of global disability-adjusted life-years as well as 3.8% of all global deaths reported in 2009 (Rehm et al., 2009), is one of the most prominent risk factors to health in the world. Alcoholic liver disease (ALD), the primary clinical manifestation of chronic alcohol abuse, has become one of the major liver diseases in China with the second highest incidence just ranking after virus hepatitis (Zhang, 2003). In Europe, almost all heavy drinking appear fatty liver, 10–20% of which progressed to severe liver cirrhosis (Breitkopf et al., 2009). Although alcohol is the only etiology of ALD, the underlying mechanism is complicated. Recently, oxygen free radicals especially hydroxyl radical with most toxic effect involved in iron-mediated ALD have attracted intensive attention (Harrison-Findik et al., 2007). What is more, it is well known that only redox-active Fe^{2+}, a small portion of intracellular iron, initiates the propagation of excess concentration of free radicals by participating in Fenton/Haber–Weiss reaction cycle (Jomova and Valko, 2011). Hence, excessive "free" iron seems to be the reliable parameter of iron toxicity.

The forms of "free" Fe^{2+} include non-transferrin bound iron (NTBI) originally identified by Hershko et al. (1978), and labile iron pool-Fe (LIP-Fe), an intracytosolic equivalent of NTBI, first proposed by Greenberg and Wintrobe (1946). Such fraction of

Abbreviations: ALD, alcoholic liver disease; DMT1, divalent metal transporter 1; Ft, ferritin; HIF-1/2α, hypoxia-inducible factor-1/2 alpha; LIP-Fe, labile iron pool-Fe; NTBI, non-transferrin bound iron; PKA, protein kinase A; TfR1, transferrin receptor 1; TRPML1, transient receptor potential mucolipin 1; ZIP14, zinc transporter member 14.

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redox-active iron represents "free" iron not linked to the major transferring and storing proteins but weakly and loosely bound to diverse low-molecular weight organic chelators (Brissot et al., 2012). Divalent metal transporter 1 (DMT1) and zinc transporter member 14 (ZIP14) are the two major carriers involved in NTBI uptake by hepatocytes (Liuzzi et al., 2006; Shindo et al., 2006). Transient receptor potential mucolipin 1 (TRPML1) is implicated in the release of iron from late transferrin receptor 1 (TfR1) in endosomes and iron complexes especially ferritin (Ft) in lysosomes, which has been recognized as the major source of cellular LIP-Fe (Dong et al., 2008). Abnormal serum NTBI has been observed in alcoholism (De Feo et al., 2001). The potential role of these specific proteins involved in the uptake and release of "free" iron, however, has remained uncertain in alcohol-induced iron overload and subsequent liver damage.

Quercetin, one of the most common flavonoids, distributes ubiquitously in plant kingdom including vegetables, fruits, herbal medicine and red wine (Boots et al., 2008). The quercetin protection against ALD has been reported, which is based on or implicated in its prominent anti-oxidative properties (Chen, 2010; Yao et al., 2009). What is more, unique structure with poly-phenolic hydroxyl groups imparts quercetin the same iron-chelating activity as classic deferoxamine clinically available for the treatment of hemochromatosis (Mladenka et al., 2011). The iron-chelating capacity of quercetin has been assumed to contribute to the extensive bioactivity despite limited bioavailability (Leopoldini et al., 2006; Manach et al., 2005), suggesting that quercetin may potentially prevent ethanol-induced "free" iron disorder and accordingly alleviate oxidative stress. However, little attention has been focused on the effect of quercetin on "free" iron in vivo in spite of studies (Zhang et al., 2011, 2006) which showed that quercetin prevented mouse iron overload with determination of hepatic total iron content without "free" iron measurements. Hence, we designed this study to investigate the effect of chronic ethanol exposure and quercetin intervention on liver damage, focusing on "free" iron and the potential molecules for serum NTBI uptake and cellular LIP-Fe release.

2. Materials and methods

2.1. Chemicals and materials

Ethanol and carbonyl iron powder were purchased from Zhenxing Chemical Factory (Shanghai, China) and Guangzhou Yuelong Metal Powder Co., Ltd. (Guangzhou, China), respectively. Quercetin (⩾98%, HPLC), apo-transferrin, gallium chloride ($GaCl_3$), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA) and 1,1,3,3-tetraethoxypropane were procured from Sigma–Aldrich. Five-(four, six-dichlorotriazinyl) aminofluorescein (DCTAF) was obtained from Molecular Probes Inc. (Eugene, OR). Anti-GAPDH rabbit polyclonal antibody, horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (secondary antibody) were obtained from Epitomics and Cell Signal, respectively. Anti-DMT1, TfR1 or TfR2 rabbit polyclonal antibodies were provided by Santa Cruz (California, USA). Anti-ferritin rabbit polyclonal antibody was offered by Abcam (UK). Anti-TRPML1 and anti-ZIP14 rabbit polyclonal antibodies were purchased from Pierce (Washington, USA) and Sigma (Missouri, USA), respectively. Western blotting detecting reagents (ECL) and re-blot buffer was supplied by Chemicon (Temecula, CA, USA), respectively. Acrylamide, tetramethylethylenediamine (TEMED), methylene acrylamide, leupeptin, and phenylmethyl sulfonylfluoride (PMSF) were provided by Promega (USA). Assay kits for aspartate/alanine transaminase (AST/ALT) and antioxidative enzymes (superoxide dismutase/SOD) were purchased from Mindray (Shenzhen, China) and Jiancheng (Nanjing, China), respectively. Amicon Ultra Filters (30000, MWCO) were provided by Millipore (Massachusetts, USA). Other chemicals and organic solvents of analytical grade were purchased from local reagent retailer.

2.2. Animal treatment

Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). Experiments described in this study were approved by the Tongji Medical College Council on Animal Care Committee. One hundred and twelve adult male C57BL/6J mice, obtained from Sino-British Sippr/BK (Shanghai, China) with body weight of 18–20 g, were randomly divided into eight groups of 15 animals each and isocaloric pair-fed with either regular or ethanol-containing Lieber De Carli liquids diets (Beijing HFK Biosicence Co., Ltd, Beijing, China) with quecertin (100 mg/kg.bw, 10 mL/kg body weight, i.g. <12# gauge needle with 60 mm length>) and/or carbonyl iron powder (0.2%) for 15 weeks. The Lieber De Carli liquid diet used as regular food by mice, contained 8.8 mg iron/1000 mL. In order to avoid the influence of quercetin on iron absorption on the premise of simulation human diet and ensure the intake of quercetin with bitter taste, quercetin was administered by gavage about 8 am followed liquid diet feeding with 2 h interval. The ethanol content of the diet was gradually increased over an 12-day period (no ethanol for day 2, one-fourth the amount for days 3–5, half the amount for days 6–9, and two-third the amount for days 10–12) to the full amount (30% of total calories as ethanol). Body weight and food consumption were monitored once a week and daily, respectively.

Animals were kept on a regular 12:12 light dark cycle at a controlled temperature (25 ± 2 °C) and a relative humidity (65–75%). The mice were sacrificed after an overnight fasting. Serum was collected from blood by centrifuge at 3500*g* for 10 min at 4 °C (Eppendorf 5810R, Hamburg, Germany). Fresh liver specimens were quickly-weighted and frozen by liquid nitrogen and then stored at −80 °C for various assays.

2.3. Determination of serum transaminases and hepatic redox status

Serum AST and ALT were measured with enzymatic kinetic method by Mindray BS-200 automatic biochemistry analyzer (Shenzhen, China) with matching kits according to the manufacturer's instructions.

Liver homogenate with isotonic saline (10%) was centrifuged at 3500×*g* for 10 min (4 °C) to prepare the supernatant. Methods of reduced glutathione (GSH) measurement was determined by the method of Moron et al. (1979), based on its reaction with 5,5′-dithiobis-2-nitrobenzoic acid to generate 2-nitro-5-thiobenzoic acid. The activity of SOD was measured in the light of the disproportionating rate of superoxide radicals generated from xanthine/xanthine oxidase system, and one unit (1 U) is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Malondialdehyde (MDA) content was estimated according to the formation of thiobarbituric acid reactive components with 1,1,3,3-tetraethoxypropane as an external standard (Buege and Aust, 1978). These parameters were determined spectrophotometrically and normalized by total protein examined by using the method of Lowry et al. (1951).

Hepatic ROS assay was based on the oxidation of dihydroethidium (DHE) which is freely permeable across cell membranes followed the production of ethidium bromide and subsequently yield red fluorescent product (Carter et al., 1994). In brief, fresh cross-sections (5 μm) of liver tissue were immediately incubated with 5 μM DHE at 37 °C for 15 min in a humidified chamber. Following washing with phosphate-buffered saline for 3 times, the fluorescence intensity was observed with Nikon 2000S fluorescence microscope (Melville, NY) and analyzed by Image-Pro Plus software.

2.4. Assay of serum NTBI and hepatic total iron and LIP-Fe

Serum NTBI was measured using fluorescein-labeled apo-transferrin (Fl-aTf) which quenched fluorescence upon binding Fe^{2+} according to the method published by Breuer and Cabantchik (2001). Briefly, the serum was mixed with reagent A (HEPES-buffered saline (HBS) containing 0.6 μM Fl-aTf, 10 mM sodium oxalate, and 0.1 mM $Gacl_3$) or reagent B (reagent A with 25 μM aTf). The fluorescence intensity in the presence of Regent A or B was recorded by a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) at 485/538 nm as excitation/emission wavelength pair after 1 h of incubation in the dark at room temperature. The concentration of NTBI was calculated according to the ratio of Regent A against B and ferric nitrilotriacetate calibration curve.

Total amount of hepatic iron was determined by flame atomic absorption spectrometry (spectrAA-240FS, USA) using an iron hollow cathode lamp. The analysis of LIP-Fe was performed according to Khan et al. (1999). In brief, liver homogenates (2.5%) were prepared using 1 mM ethylenediamine tetra acetic acid (EDTA) to dissociate LIP-Fe and centrifuged at 20,000×*g* for 15 min at 4 °C. The resultant supernatants were ultra-filtrated on Micron-30 at 14,000×*g* for 20 min at 4 °C for atomic absorption spectrophotometric assay as LIP-Fe.

2.5. Expression of mRNA and protein

The expression of mRNA was based on the real-time quantitative PCR analysis. Briefly, total RNA was extracted from liver using the TRIzol® reagent and target mRNAs expressions were quantified by 7900HT PCR machine (Applied Biosystems, Forster, CA) with SYBR green-based qRT-PCR kit (TaKaRa BIO INC, Dalian) and specific primers. The efficiency of the PCR reactions and specificity of the product were determined using a series of dilutions of a standard hepatic sample and melting curve analysis, respectively. The relative mRNA expression was assayed by using